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APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A  
FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/111,794

FILING DATE: December 11, 1998

## PRIORITY DOCUMENT

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## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)					
Given Name (first and middle (if any))		Family Name or Surname		Residence (City and either State or Foreign Country)	
Robert		Shipman		Mississauga, Canada	
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
Method and Kit for the Characterization of Antibiotic-Resistance Mutations in Mycobacterium tuberculosis					
CORRESPONDENCE ADDRESS					
Direct all correspondence to:					
<input type="checkbox"/> Customer Number		<input type="text"/>			
OR Type Customer Number here					
<input type="checkbox"/> Firm or Individual Name		Oppedahl & Larson, LLP			
Address		PO Box 5270			
Address		611 Main Street			
City		Frisco	State	CO	ZIP 80443-5270
Country		US	Telephone	970 668 2050	Fax 970 668 2082
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		20		<input type="checkbox"/> Small Entity Statement	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		2		<input type="checkbox"/> Other (specify) <input type="text"/>	
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees				FILING FEE AMOUNT (\$)	
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 15-0610				150.00	
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,

SIGNATURE

*Marina T. Larson*

Date

12/11/98

TYPED or PRINTED NAME

Marina T. Larson

REGISTRATION NO.

32,038

(if appropriate)

Docket Number:

V6ENP055M

TELEPHONE

970 668 2050

### USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

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# FEE TRANSMITTAL

Patent fees are subject to annual revision on October 1.  
These are the fees effective October 1, 1997.  
Small Entity payments must be supported by a small entity statement,  
otherwise large entity fees must be paid. See Forms PTO/SB/09-12.  
See 37 C.F.R. §§ 1.27 and 1.28

## Complete if Known

Application Number	TO BE ASSIGNED
Filing Date	11 DECEMBER 1998
First Named Inventor	SHIPMAN, ROBERT
Examiner Name	N/A
Group / Art Unit	N/A
Attorney Docket No.	VGENP055PV

TOTAL AMOUNT OF PAYMENT (\$) 150.00

## METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:  
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2. ☒ Payment Enclosed:  
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## FEE CALCULATION

### 1. BASIC FILING FEE

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
101 790	201 395	Utility filing fee	
106 330	206 165	Design filing fee	
107 540	207 270	Plant filing fee	
108 790	208 395	Reissue filing fee	
114 150	214 75	Provisional filing fee	150
SUBTOTAL (1) (\$)			150

### 2. EXTRA CLAIM FEES

Total Claims	Extra Claims	Fee from below	Fee Paid
Independent Claims	-20** =	X	
Multiple Dependent Claims	-3** =	X	

\*\*or number previously paid, if greater; For Reissues, see below

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description
103 22	203 11	Claims in excess of 20
102 82	202 41	Independent claims in excess of 3
104 270	204 135	Multiple dependent claim, if not paid
109 82	209 41	** Reissue independent claims over original patent
110 22	210 11	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$)

## FEE CALCULATION (continued)

### 3. ADDITIONAL FEES

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
105 130	205 65	Surcharge - late filing fee or oath	
127 50	227 25	Surcharge - late provisional filing fee or cover sheet	
139 130	139 130	Non-English specification	
147 2,520	147 2,520	For filing a request for reexamination	
112 920*	112 920*	Requesting publication of SIR prior to Examiner action	
113 1,840*	113 1,840*	Requesting publication of SIR after Examiner action	
115 110	215 55	Extension for reply within first month	
116 400	216 200	Extension for reply within second month	
117 950	217 475	Extension for reply within third month	
118 1,510	218 755	Extension for reply within fourth month	
128 2,060	228 1,030	Extension for reply within fifth month	
119 310	219 155	Notice of Appeal	
120 310	220 155	Filing a brief in support of an appeal	
121 270	221 135	Request for oral hearing	
138 1,510	138 1,510	Petition to institute a public use proceeding	
140 110	240 55	Petition to revive - unavoidable	
141 1,320	241 660	Petition to revive - unintentional	
142 1,320	242 660	Utility issue fee (or reissue)	
143 450	243 225	Design issue fee	
144 670	244 335	Plant issue fee	
122 130	122 130	Petitions to the Commissioner	
123 50	123 50	Petitions related to provisional applications	
126 240	126 240	Submission of Information Disclosure Stmt	
581 40	581 40	Recording each patent assignment per property (times number of properties)	
146 790	246 395	Filing a submission after final rejection (37 CFR 1.129(a))	
149 790	249 395	For each additional invention to be examined (37 CFR 1.129(b))	
Other fee (specify) _____			
Other fee (specify) _____			
* Reduced by Basic Filing Fee Paid			
SUBTOTAL (3) (\$)			

## SUBMITTED BY

Typed or Printed Name MARINA T. LARSON  
Signature Marina T. Larson

Date 12/11/98

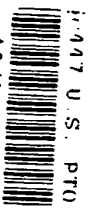
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*JP/Prov*

12/11/98



TRANSMITTAL FORM

U.S. Application No. : To be assigned  
Filing Date : December 11, 1998  
Application of: : Shipman, Robert  
For: : Method and Kit for the  
: Characterization of Antibiotic-  
: Resistance Mutations in  
: Mycobacterium  
Attorney Docket No. : VGENP055PV

Enclosures:

- Provisional Patent Application Cover Sheet (PTO/SB/16)
- Fee Transmittal (PTO/SB/27)
- Check No. ~~004264~~ for \$150.00 *Ch. No. 004549*
- Specification - 20 pages
- Drawings - 2 pages

Date: December 11, 1998

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CERTIFICATE OF MAILING

CERTIFICATE OF EXPRESS MAIL UNDER 37 C.F.R. § 1.10

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Mary Ann Healey

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*Mary Ann Healey*  
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**METHOD AND KIT FOR THE CHARACTERIZATION OF  
ANTIBIOTIC-RESISTANCE MUTATIONS IN  
*MYCOBACTERIUM TUBERCULOSIS***

**Background**

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

*M. tuberculosis* can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing its uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for *M. tuberculosis*, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular

biological methods for the detection of *M. tuberculosis* and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR-based or probe-based. These tests are used primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. Tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 504-531 <sup>a</sup>
2.	Isoniazid	katG gene	codon 275/315/328 <sup>b</sup>
3.	Isoniazid	fabG gene	unknown <sup>c</sup>
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	codon 541-619 <sup>d</sup>
5.	Azithromycin	23S rRNA sequence nucleotide 2568A <sup>e</sup>	
6.	Pyrazinamide	pncA gene	codon 47/85 <sup>f</sup>
7.	Ethambutol	embB gene	codon 306 <sup>g</sup>
8.	Streptomycin	rpsL/s12 gene	codon 43/88 <sup>h</sup>

- |     |               |                  |                          |
|-----|---------------|------------------|--------------------------|
| 9.  | Streptomycin  | 16S/rrs sequence | unknown <sup>i</sup>     |
| 10. | Ciprofloxacin | gyrA gene        | codon 88-95 <sup>j</sup> |

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in its present form.

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical *M. tuberculosis* sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in *M. tuberculosis*.

#### Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the rpoB (rifampin), katG (isoniazid), oxyR-ahpC PR (isoniazid), fabG (isoniazid), rpsL/s12 (streptomycin), 16S/rrs (streptomycin), embB (ethambutol), pncA (pyrazinamide), gyrA (ciprofloxacin) and 23S (azithromycin) genes. Using these primer sets and the OpenGene™ automated DNA sequencing system, a protocol has been developed which permits both the rapid identification of *M. tuberculosis* and the detection of antibiotic resistance-associated mutations in a series of gene targets. The

present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture *M. tuberculosis* and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

#### DESCRIPTION TO THE FIGURES

Fig. 1 shows known testing protocols for *M. tuberculosis*; and

Fig. 2 shows a hierarchical assay scheme for evaluating *M. tuberculosis* type in accordance with the invention.

#### DETAILED DESCRIPTION OF THE INVENTION

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are known, some of which require prior amplification and some of which

do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

### Primers

#### rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220 5' TAC GGT CGG CGA GCT GAT CC 3'

rpoB-R amplification primer, 20-mer, bp2611-2592 5' TAC GGC GTT TCG ATG AAC CC 3'

rpoB-5s sequencing primer, 20-mer, bp2201-2220 5' TAC GGT CGG CGA GCT GAT CC 3'

rpoB-3s sequencing primer, 20-mer, bp2611-2592 5' TAC GGC GTT TCG ATG AAC CC 3'

```
2161 aaaccgaaga catcgaccac ttccggcaacc gccgcctgcg tacggtcggc gaactgaccc
2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg
2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaaccgcg
2401 tgtcgggggt gaccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg
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2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgccga  
2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggtc gctgtcggg tacgcgcggg  
2581 tcaaccggtt cggttcac gaaacgccgt accgcaaggt ggtcgacggc gtggtagcg

#### **katG (isoniazid resistance)**

katG-F amplification primer, 20-mer, bp722-741 5' ATG GGG CTG ATC TAC GTG AA 3'

katG-R amplification primer, 20-mer, bp1250-1231 5' GGT GTT CCA GCC AGC GAC GC 3'

katG-5s sequencing primer, 20-mer, bp722-741 5' ATG GGG CTG ATC TAC GTG AA 3'

katG-3s sequencing primer, 20-mer, bp1250-1231 5' GGT GTT CCA GCC AGC GAC GC 3'

661 gtcggcgat gagcggtaca gcggttaagcg ggatctggag aaccgctgg ccgcggtgca  
721 gatgggctg atctacgtga accggagg gccgaacggc aaccggacc ccatggcgc  
781 ggcggtcgac attcgcgaga cgttcggcg catggccatg aacgacgtcg aaacagcggc  
841 gctgacgtc ggcggtcaca ctttcggtaa gacccatggc gccggcccg ccgatctggt  
901 cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta  
961 tggcaccgga accggttaagg acgcgatcac cagcggtac gaggtcgtat ggacgaacac  
1021 cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac  
1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat  
1141 cccggaccg ttcggcgggc caggcgctc cccgacgatg ctggccactg acctctcgt  
1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt  
1261 ggccgacgag ttccccaagg cctggtacaa gctgatccac cgagacatgg gtccegttgc

#### **oxyR-ahpC intergenic region (PR)**

PR-F amplification primer, 20-mer, bp451-470 5' ACC ACT GCT TTG CCG CCA CC 3'

PR-R amplification primer, 20-mer, bp687-668 5' CCG ATG AGA GCG GTG AGC TG 3'

PR-5s sequencing primer, 20-mer, bp451-470 5' ACC ACT GCT TTG CCG CCA CC 3'

PR-3s sequencing primer, 20-mer, bp687-668 5' CCG ATG AGA GCG GTG AGC TG 3'

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgaac gtcgactggc  
421 tcatatcgag aatgcttgcg gcaactgctga accactgctt tgccgccacc gcggcgaacg  
481 cgcgaaagccc ggccacggcc ggctagcacc tcttgccggc gatgccgata aatatgggtg  
541 gatatatcac ctttgccctga cagcgacttc acggcacgat ggaatgtcgc aaccaaagtc  
601 attgtccgct ttgatgatga ggagagtcac gccactgcta accattggcg atcaattccc  
661 cgctaccag ctcaccgctc tcatcgccg tgacctgtcc aaggtcgacg ccaagcagcc  
721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc ggggtggtgt

fabG (isoniazid resistance)

fabG-F amplification primer, 20-mer, bp56-75 5' CCT CGC TGC CCA GAA AGG GA 3'

fabG-R amplification primer, 20-mer, bp303-284 5' ATC CCC CGG TTT CCT CCG GT 3'

fabG-5s sequencing primer, 20-mer, bp56-75 5' CCT CGC TGC CCA GAA AGG GA 3'

fabG-3s sequencing primer, 20-mer, bp303-284 5' ATC CCC CGG TTT CCT CCG GT 3'

1 agcgcgacat acctgctgcg caattcgtag ggcgtcaata caccgcagc caggccctcg  
61 ctgcccagaa agggatccgt catggctcgaa gtgtgctgag tcacaccgac aaacgtcacg  
121 agcgtaaccc cagtgcgaaa gttcccgccg gaaatcgag ccacgttacg ctctgtggaca  
181 taccgatttc ggcccggccg cggcgagacg atagggtgtc ggggtgactg ccacagccac  
241 tgaagggggc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg  
301 gacgggctg gcgatcgac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20 5' CGG TAG ATG CCA ACC ATC CA 3'

s12-R amplification primer, 20-mer, bp384-365 5' GCA TCA GCC CTT CTC CTT CT 3'

s12-5s sequencing primer, 20-mer, bp1-20 5' CGG TAG ATG CCA ACC ATC CA 3'

s12-3s sequencing primer, 20-mer, bp384-365 5' GCA TCA GCC CTT CTC CTT CT 3'

1 cgqtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag  
61 gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac  
121 accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgct gaagttgacg  
181 agtcaggctc aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg  
241 atggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc  
301 cgcggttcgc tggatacgca ggggtgtcaag aaccgcaaac aggcacgcag ccgttacggc  
361 gctaagaagg aqaagggtg atgccacgca aggggcccg gccaagcgt ccgttggtca

#### 16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25 5' GGT GAT CTG CCC TGC ACT TCG 3'

16S-R amplification primer, 21-mer, bp147-127 5' CGT CAC CCC ACC AAC AAG CTG 3'

16S-5s sequencing primer, 21-mer, bp5-25 5' GGT GAT CTG CCC TGC ACT TCG 3'

16S-3s sequencing primer, 21-mer, bp147-127 5' CGT CAC CCC ACC AAC AAG CTG 3'

1 cgtgggtgat ctgccttcca cttcgggata agcctgggaa actgggtcta ataccggata  
61 ggaccacggg atgcatgtct tgtgggtgaa agcgctttag cgggtgtggga tgagcccgcg  
121 gcctatcagg ttgttgggtg ggtgacg

#### embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781 5' CGG CAA GCT GGC GCA CCT TCA 3'

embB-R amplification primer, 21-mer, bp8040-8020 5' AGC CAG CAC ACT AGC CCG GCG 3'

embB-5s sequencing primer, 21-mer, bp7761-7781 5' CGG CAA GCT GGC GCA CCT TCA 3'

embB-3s sequencing primer, 21-mer, bp8040-8020 5' AGC CAG CAC ACT AGC CCG GCG 3'

7741 cggcatgcgc cggtgatgc cggcaagctg ggcaccttc acctgaccg acgctggt  
7801 gatattcggc ttctgtctt ggcattgcat cggcgcgaat tcgtcggacg acggtacat  
7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgtggt  
7921 cggcagcccg gaggatccct tcggtggta ttacaacctg ctggcgtga tgacccatgt  
7981 cagcgacgcc agtctgtgga tgcgctgcc agacctggc gcccggctag tctactgct

**pncA (pyrazinamide resistance)**

pncA-F amplification primer, 20-mer, bp1-20 5' ATG CGG GCG TTG ATC ATC GT 3'

pncA-F amplification primer, 20-mer, bp561-542 5' TCA GGA GCT GCA AAC CAA CT 3'

pncA-5s sequencing primer, 20-mer, bp1-20 5' ATG CGG GCG TTG ATC ATC GT 3'

pncA-3s sequencing primer, 20-mer, bp561-542 5' TCA GGA GCT GCA AAC CAA CT 3'

1 atgccccgct tcatcatcgt cgacgtgcag aacgacttct gcgaggggtg ctgctggcg  
61 gtaaccgggtg gcgcgcgct ggcgcgcgc atcagcgact acctggccga agcgcgccgac  
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc  
181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgccgac  
241 ttccatccca gtctggacac gtccgcaatc gaggcgggtg tctacaaggg tgcctacacc  
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg  
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc  
421 cagacggccg aggacgcggt acgcaatggc ttggccacca ggggtgctgg ggacctgaca  
481 gcgggtgtgt cggccgatac caccgtcgcc gcgtggagg agatgcgcac cgccagcgtc  
541 gacttgggtt gacgtcctg a

**gyrA (fluoroquinilone/ciprofloxacin resistance)**

gyrA-F amplification primer, 20-mer, bp2383-2402 5' CAG CTA CAT CGA CTA TGC GA 3'

gyrA-R amplification primer, 20-mer, bp2702-2683 5' GGG CTT CGG TGT ACC TCA TC 3'

gyrA-5s sequencing primer, 20-mer, bp2383-2402 5' CAG CTA CAT CGA CTA TGC GA 3'

gyrA-3s sequencing primer, 20-mer, bp2702-2683 5' GGG CTT CGG TGT ACC TCA TC 3'

2341 cgaccggatc gaaccgggtg acatcgagca ggagatgcag ccagctaca tcgactatgc  
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca  
2461 tcgccgggtg ctctatgcaa tgttcgattc cggcttcgc cgggaccgca gccacgcca  
2521 gtccggcccg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat  
2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctaccgc tgggtggacgg  
2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg ggatgaggt acaccgaagc  
2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

**23S (macrolide/azithromycin resistance)**

23S-F amplification primer, 20-mer, bp2444-2463 5' CGA AAT TCC TTG TCG GGT AA 3'

23S-R amplification primer, 20-mer, bp2683-2664 5' GTA TTT CAA CAA CGA CTC CA 3'

23S-5s sequencing primer, 20-mer, bp2444-2463 5' CGA AAT TCC TTG TCG GGT AA 3'

23S-3s sequencing primer, 20-mer, bp2683-2664 5' GTA TTT CAA CAA CGA CTC CA 3'

2401 gccccagtaa acggcgggtg taactataac catcctaagg taggaaatt ccttgcggg  
2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg  
2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccgga  
2581 ccttcactac aacttggtat tgggtgttcgg tacggtttgt gtaggatagg tgggagactt  
2641 tgaagcacag acgccagttt gttggagtc gttgttgaat taccactctg atcgtattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OpenGene sequencer), at least one of the sequencing primers is preferably labeled with a fluorescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as Cy5.0 or Cy5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each

resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers), and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix		1 PCR	10 PCRs	final conc. / PCR
genomic DNA	(20ng/ul)	1.0ul		20ng (~0.5fM)
10X PCR buffer I		2.5ul	25.0ul	1X
2.5mM dNTP mix	(1:1:1:1)	2.5ul	25.0ul	250uM
DMSO		1.3ul	13.0ul	5%
Taq DNA polymerase (1U)		0.2ul	2.0ul	1 unit
molecular grade water		16.5ul	165.0ul	
<u>MTB gene primers</u>	(10uM)	<u>1.0ul</u>	10.0ul	10pmol per primer
total volume per PCR		25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles

.	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

#### Cycle sequencing mastermix

rpoB template	2.0ul
10X VGI Sequenase™ buffer	2.5ul
DMSO	3.5ul

2.5uM dye-sequencing primer	2.0ul
PCR grade water	9.0ul
<u>1:10 diluted Thermosequenase™</u>	<u>3.0ul</u>
total volume	22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the Clipper™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The Clipper™ sequencer is set-up as described in the *OpenGene Automated DNA Sequencing System User Manual*. Run parameters for the Clipper™ sequencer are 54°C / 1300volts / 0.5sec sampling / 35min run / 50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with Cy5.0 and Cy5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GeneLibrarian™. This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GeneLibrarian™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A

limitation of the AFB stain methodology is its limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect *M. tuberculosis* (*rpoB*), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat *M. tuberculosis* infections (*rpoB*, *katG*, *rpsL/s12*, 23S, PR, *embB*, *pncA*, *gyrA*) and detect other species of mycobacteria (23S) in the absence of *M. tuberculosis* (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (*rpoB*), isoniazid (*katG*), streptomycin (*rpsL/s12*) and azithromycin (23S). In addition the *rpoB* amplification indicates the presence of *M. tuberculosis* and in the absence of *rpoB* amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (*embB*), pyrazinamide (*pncA*) and ciprofloxacin (*gyrA*). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of *M. tuberculosis* from both AFB smear-

positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

#### Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada, Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the *rpsL*/*s12* gene in four isolates. Parallel antibiotic resistance-associated mutations in the *rpoB* (rifampin), *katG* (isoniazid), *PR* (isoniazid), *embB* (ethambutol), *pncA* (pyrazinamide) and *gyrA* (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference.

- <sup>a</sup> DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* 38: 2380-2386.
- <sup>b</sup> WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. *Antimicrob Agents Chemother* 41: 1601-1603.
- <sup>c</sup> S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities. *Antimicrob Agents Chemother* 41: 600-606.
- <sup>d</sup> A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at the reference laboratory level. *Antimicrob Agents Chemother* 35: 719-723.
- <sup>e</sup> C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J Appl Microbiol* 83: 634-640.
- <sup>f</sup> C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J Appl Microbiol* 83: 634-640.
- <sup>g</sup> MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 41: 2629-2633.
- <sup>h</sup> A Scorpio et al. (1997). Characterisation of pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 41: 540-543.

- <sup>i</sup> C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. *J Infect Disease* 174: 1127-1130.
- <sup>j</sup> KA Nash et al. (1995). Genetic basis of macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. *Antimicrob Agents Chemother* 39: 2625-2630.

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Table 1

gene (antibiotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
ipob (rifampin)	cac526tac, His526Tyr	tcg553ttg, Ser553Leu	cac526gac, His526Asp	tcg553ttg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpSL/s12 (streptomycin)	wt	aag43aagg, Lys43Arg	aag43aagg, Lys43Arg	aag88aagg, Lys88Arg	aag43aagg, Lys43Arg
16s/rfs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	gtc292ttc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tc65tct, Ser65Ser	wt	att1133aat, lle1133asn	wt	tc65tct, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

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Figure 1.

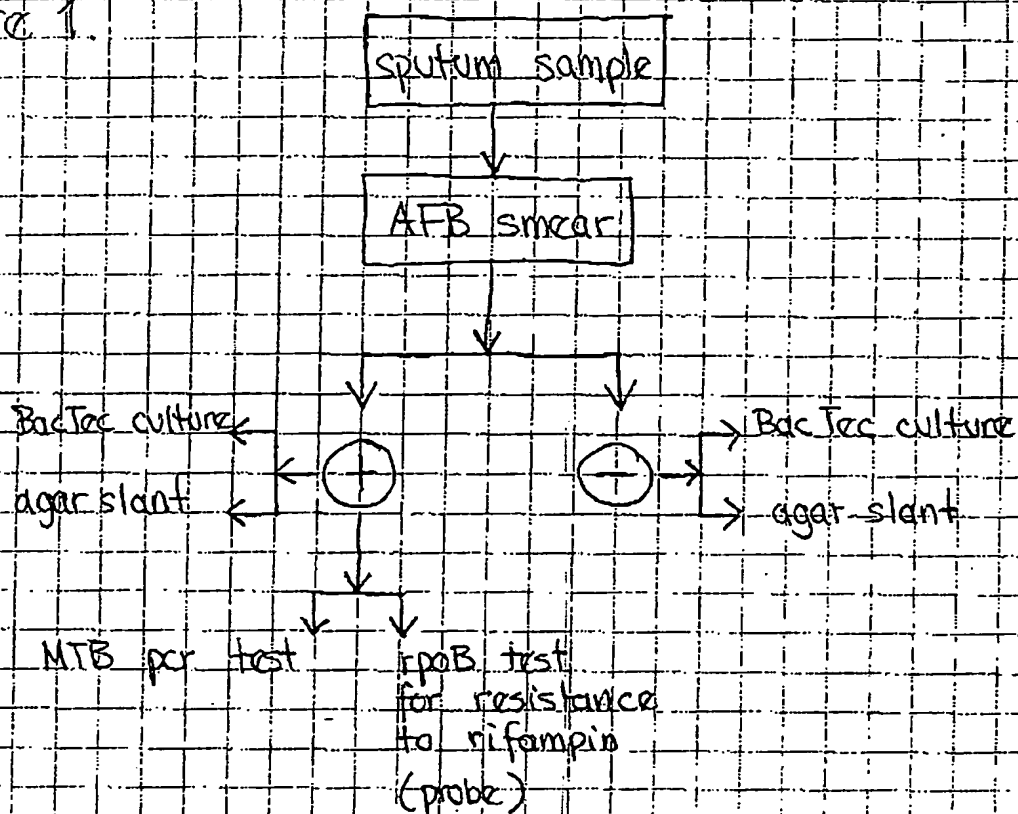


Figure 2

sputum sample

AFB smear

Bactec culture  
agar slant

Bactec culture  
agar slant



Group I

MTB  
NTB  
MTB  
MTB  
rpoB  
katG  
rpsL/s12  
23S

MTB  
NTB  
NTB  
NTB  
rpoB  
katG  
rpsL/s12  
23S

Group II

MTB  
NTB  
NTB  
NTB  
PR  
embB  
pncA  
gyrA

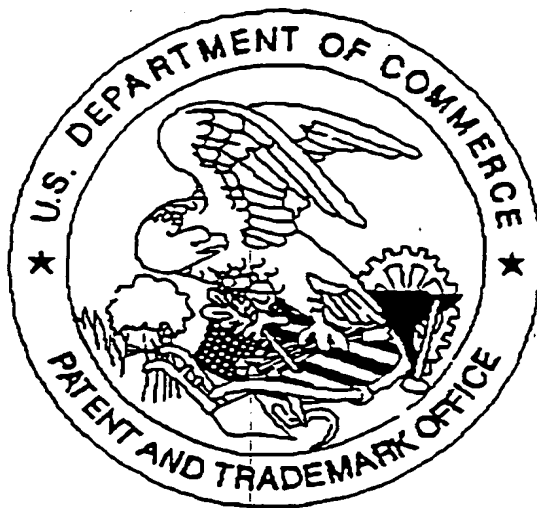
MTB  
NTB  
NTB  
NTB  
PR  
embB  
pncA  
gyrA

Group III

MTB  
NTB  
16S/rrs  
fabG

MTB  
NTB  
16S/rrs  
fabG

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